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A CONTRIBUTION TO OUR KNOWLEDGE OF THE FUNCTION OF THE ŒNOCYTES OF INSECTS.[1]

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The function of the cells known as the œnocytes has been the object of a considerable amount of speculation by various investigators. The studies which constitute the basis of these speculations have all been of a morphological character and, while valuable in their way, throw no light on the physiology of these very singular elements. So much has been written about them, especially about their morphology, that we will consider only a few of the more interesting views on their possible function. In 1873 Graber called attention to the fact that the œnocytes are glands secreting a substance concerning which nothing is known. Later ('91) he supposed that they are metamorphosed into the fat-body, and also give rise to blood corpuscles. This was corrected by Wheeler, in 1891, who through a study of the embryological development of these cells, concluded that they neither give rise to the fat-body nor to the blood. Pantel ('98) and Berlese ('99) endowed the œnocytes with an excretory function, the latter supposing that they serve during the periods of moulting and pupation when the Malpighian tubules are functionless. Anglas ('00) advanced the view that the œnocytes may possibly secrete ferments. Koschevnikov ('00) makes the remarkable statement, among others, that he has preparations which show plainly that the œnocytes swallow fat cells. He further says, "unnecessary substances which get into the blood stop in the interior of the œnocytes in the form of granules. The cells are not periodically emptied of this excretion product, but

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are continually being filled. Finally they are completely filled and are made entirely useless for further activity. It may be that the inability of the œnocytes for further work brings about a disturbance of the regular metabolism, and is hence one of the causes of the sinking life activity of the insect." It seems as if Koschevnikov must have confused the œnocytes with phagocytes; at any rate he did not study the œnocytes. Janet ('07) regards the œnocytes as real unicellular glands. "Like these they take from the blood elements for their functional activity. The substance resulting from this activity they give up by osmosis to the adipocytes which use it possibly for the elaboration or dissolution of reserves, possibly for the production of a special reserve substance." Verson ('11) found at times at the periphery of the cells microscopical exudations of a particular excretion which was accompanied by changes in the form and size of the nucleus. Gee ('11) injected specimens of *Platyphylax designatus* larvæ with methylene blue, and found that immediately after injection the œnocytes and spinning glands both took the stain, the œnocytes less than the spinning glands. "The Malpighian tubules, except in one case, did not take the stain. In larvæ killed half an hour after injection the œnocytes were more deeply stained, but no coloration was observed in the Malpighian tubules. Larvæ killed one hour after injection showed that the Malpighian tubes had begun to take up the blue color, but that the œnocytes and spinning glands were becoming less intense, in coloration." He reached the conclusion that the similar reaction of œnocytes and spinning glands towards methylene blue seems to show that the œnocytes are secretory rather than excretory, the spinning glands being secretory. "The nature of their secretion is difficult and practically impossible to determine. Can it be that their function is the secretion of a substance or enzyme which is of aid to the fat-body in its constructive work?"

It will be seen from this short review that there are many and diverse opinions as to the function of the œnocytes. On reviewing the literature on the morphology of these cells, the divergence of views is seen to be still greater, no two people agreeing in more than a few points. The insects used by the

various investigators were different species and very often insects belonging to distantly related orders. Of course, the form and body orientation of the œnocytes is quite different in non-related forms and even in members of the same species at different periods of their life history. This is exactly what we should expect to find. In numerous cases, however, the varied opinions can be attributed to the fact that many of the investigators did not study the same cells.

I have made sections of larvæ of Trichoptera, Lepidoptera and Diptera and find that the differences between their œnocytes are merely differences of size, shape, density of granulation and amount of ramification of the nuclei. Generally speaking the œnocytes are large, yellow, more or less isolated cells, so large in fact that in some forms they can be readily identified with a pocket lens. They are located in the abdominal segments and in such only as bear spiracles. Here the œnocytes are situated behind the tracheæ. They do not seem to be definitely attached to the tracheæ and sections do not reveal the intrusion of tracheal filaments into these cells. A cytological study with the orange G and iron hæmatoxylin method shows the cytoplasm to be finely granular and the nucleus to be greatly enlarged and ramified, giving the cells the appearance of being highly active.

To throw any light on the physiology of these cells was a difficult task. Comparatively speaking, they are large, yet in nearly all forms too small to deal with experimentally. To be certain of any test one has to have a bulk of material and further after obtaining it, one must be able to dissect out the organs with ease and be certain at all times that they are the same. Fortunately Mr. James W. Chapman, entomologist of the city of Boston, called my attention to the larvæ of the leopard moth (*Zeuzera pyrina*). The life history of this caterpillar, as worked out by Mr. Chapman, extends over a period of three years, during which time it feeds on practically every species of tree or shrub except conifers, and attains at the end of the third year a very large size, accumulating a great amount of fatty tissue and consequently becoming very heavy. On dissecting some of these larvæ, I found the œnocytes to be so enormous that in a three-year-old larva, a cluster could be recognized with the naked

eye. In one and two year old larvæ they are much smaller, for they grow in size with age as do the other organs. In this species, the œnocytes are located in the seven abdominal segments which are just those which bear the abdominal spiracles. Behind these they are situated, occurring in clusters around the tracheæ. There are only two clusters to a segment, one on each side. The number of cells in a cluster varies, from ten to forty or fifty, in different segments. The size also varies considerably in different segments (diameter 175–250 μ) and even in the same cluster, yet the smallest one far surpasses the size of any other cell within the body of the insect.

Through the kindness of Mr. Chapman, I was able to obtain a large amount of material and, following the suggestion of some of the previous investigators, principally Anglas and Janet, I began to work on the hypothesis that the œnocytes are unicellular glands, perhaps secreting a ferment.

A number of tests were made for lipase and oxydase. First, however, to become thoroughly acquainted with the reactions of these enzymes, the pancreases of four hogs were used. Lipase and oxydase are known to occur in these organs, and it was thought advisable to work with them for a time before applying tests to the œnocytes. The pancreas was cut from the hog immediately after it was slaughtered, so as to be certain that it was quite fresh. An extract of the organ was at once preserved in toluol to keep out all bacterial infection. This extract was then diluted with physiological .65 per cent. salt solution, one part of extract to ten parts of saline solution. To two cubic centimeters of this diluted extract one fourth of a cubic centimeter of ethyl butyrate was added, plus a small quantity of lacmoid solution. Purified lacmoid crystals were used and the solution was made as nearly neutral as possible. The extract was, of course, kept under the layer of toluol while the other reagents were being added. The specimen was then put in an incubator at body temperature and kept there for 24 hours. As a control test a second and aliquot portion of pancreatic juice was boiled in order to kill any enzyme. This was treated with the same reagents in exactly the same manner, and put in an incubator for 24 hours. At the end of this time both specimens

were taken out and it was found that the liquid in the test tube containing the live enzyme had turned red. The fat-splitting enzyme (lipase) had split the ethyl butyrate into alcohol and butyric acid. The control test retained its former blue tint, namely, that of the lacmoid.

The next thing was to see with how small a quantity of extract and reagents the reaction could be obtained. For this purpose very small glass tubes of equal sizes were blown. For the liquids eye droppers of equal sizes were used. A drop was sucked into the dropper and the glass was graded into four equal parts, so that it was possible by having droppers of exactly the same size and gradation to use for each test the same amounts of liquid. The amounts of the substances were then decreased until two drops of pancreatic extract and one fourth of the amount of one drop of ethyl butyrate plus the lacmoid solution were used. The toluol was never omitted. Control tests were again made each time. The characteristic red color was obtained.

When I was thoroughly satisfied that I had mastered the reaction, I made an extract from the œnocytes of large leopard moth larvæ. Two caterpillars, each measuring about $1\frac{3}{4}$ inches, were used for each experiment. All of the œnocytes were dissected out in physiological salt solution and rubbed in a very small agate mortar. The extract from the œnocytes plus a small quantity of saline solution which was added equalled two drops. This was taken without any further dilution and, accompanied by control tests, treated in exactly the same way as the small amounts of pancreatic extract had been treated. Six experiments were performed. In not one case did I get an acid reaction. This seems to indicate that lipase or a fat-splitting enzyme is not present in the œnocytes.

It occurred to me that perhaps the fat of insects might be different from vertebrate fat, and that after all the œnocytes might secrete a ferment of some sort, the presence of which the above reagents would not reveal and which might have the power of splitting this fat. I could find in the literature on fats, nothing but the broad statement that all animal fats are triglycerides of oleic, stearic and palmitic acids. The important question for me was therefore to determine whether or not insect

fats are triglycerides. This was easily accomplished by making the acrolein test. Fatty tissue was dissected out of larvæ and crushed and the fat extracted with ether. This was filtered and evaporated. An amount of the evaporated fat was then heated gently with some potassium acid sulphate till vapors appeared. These were smelled and the nasty odor of acrolein was at once detected. Control tests were made with lard. Hence, I think, I am justified in saying that insect fats are like other animal fats, and further think it safe to say that since insect fats are triglycerides like vertebrate fats, a fat-splitting enzyme like lipase would react to the reagents used for determining that enzyme in vertebrate fats. It must also be considered that a simple ester like ethyl butyrate is chosen for the test on account of the ease with which even the weakest lipase will decompose it. It is therefore quite reasonable to say that lipases are absent when ethyl butyrate is not acted upon.

The tests for oxidizing enzymes were far more difficult, but in the end the results were positive after the technique had been perfected.

The ordinary way of determining whether one is dealing with a peroxydase or a true oxidizing ferment is by the guajacum tincture method. This method, although repeated trials were made, gave no results so far as the œnocytes were concerned. The guajacum was never decomposed and the H_2O_2 seemed not to be acted upon. As will be seen later, the œnocyte extract really decomposed the H_2O_2, but as I had no efficient indicator, it was practically impossible to tell whether there was any reaction, although the amount of the reagent was decreased in proportion to the amount of the extract. When large amounts of extract from whole caterpillars were used, I found, as did Ostwald ('07), that very often the H_2O_2 was decomposed so violently as to cause the liquid to bubble up in the test tube. When using such large amounts of extract the guajacum is also acted upon and the "Hartzsuspension" turns blue, showing peroxydases to be present also.

The method I applied to the œnocytes and which I am about to describe, gave positive results in so far as it showed that the œnocyte extract contains oxidizing ferments to a much greater

degree than any of the other organs or tissues. I was unable to classify these oxidizing enzymes any further and am unable to say whether they are oxydases or katalases. A very delicate method had to be devised, for one must bear in mind that the amount of œnocyte extract which can be obtained even from a large number of larvæ is very small. I have already called attention to the fact that the œnocytes are very large in *Zeuzera pyrina* when compared with those of other forms, yet this statement is merely relative and it must be remembered that from the point of view of the physiological chemist they are really minute organs.

The pancreatic extract of the hog was again used and the amounts of the extract and reagents were again decreased in order to train the eye as before to light reactions. Training the eye was hardly necessary in either case. The reaction for lipase was decidedly negative while the reaction for oxidizing ferments was decidedly positive. It was thought safer, however, to take these precautions.

It might be well here to say something concerning the reagent, which was that employed by clinical workers for the demonstration of occult blood. This reagent consists of: 100 c.c. of a 20 per cent. solution of NaOH + 2 grs. phenolphthal*ein* + 10 grs. zinc dust. This is boiled slowly till the solution is decolorized. The fluid is then filtered while still hot into a colored bottle under white petroleum oil. Great care must be exercised to keep out the oxygen of the air, or it will color.

In the experiments one half the quantity of reagent was added to double the quantity of pancreatic extract and a drop or two of a 3 per cent. solution of hydrogen peroxide was added to this. Owing to the fact that oxidizing enyzmes are present in the pancreas, the solution strikes a red color. As control tests water was used instead of extract and treated in the same way. No attention was paid to change of color which develops on prolonged standing. A layer of petroleum was always kept above the specimens to exclude the air.

It was found that the above reagent was satisfactory only so long as a considerable amount of extract was used. When I came down to using very small amounts, *e. g.*, one or two drops,

the alkali was entirely too strong and prevented the appearance of a red color. Hence, it proved to be necessary to resort to a finer reagent. The phenolphtha*lin* had first to be isolated as such. A quantity of reagent for occult blood was taken, hydrochloric acid was added till the precipitate that formed had redissolved. The solution was extracted with ether which was washed 3–4 times with small quantities of water to get rid of the acid. The ether was then evaporated without heat in hydrogen. The evaporation in an indifferent gas is safer, although not absolutely necessary, for I find that the phenolphtha*lin* crystals will keep splendidly if stored in very small vials especially when they are filled to the brim before corking so as to exclude the air. Both methods were tried, however. On evaporation one obtains a crust of phenolphtha*lin* which may, if desired, be crystallized from alcohol or a mixture of ether and petroleum ether, which should evaporate spontaneously.

A small piece of phenolphtha*lin* was now placed in a drop of pancreatic extract to which a drop of a ½ per cent. KOH solution and a drop or two of a 3 per cent. solution of hydrogen peroxide had been added. All work was done under oil. The characteristic red color was immediately obtained. Control tests with water were performed each time.

The great quantity of KOH ordinarily used in making the reagent for occult blood is necessary to reduce the phenolphthal*ein* to phenolphtha*lin*. But after this is accomplished the excess of KOH must be gotten rid of as described above, otherwise the color will not develop when such infinitesimal quantities of extract have to be dealt with. A drop or two of ½ per cent. KOH solution seems to be just the proper amount and concentration to obtain the red salt.

In the last experiment with the hog extract two drops of extract were used; a crystal of phenolphtha*lin* was added and to this one drop of ½ per cent. KOH solution plus one drop of hydrogen peroxide. The characteristic red color was immediately obtained. I repeat, as this is very important, that all work was done under oil. Control tests likewise accompanied the actual tests.

Two drops of œnocyte extract were now taken, treated in

exactly the same manner as the small amounts of hog extract had been treated, and the immediate reaction was very characteristic. Eighteen experiments of this sort were performed and as all cells have catalytic ferments, other cells as the fat cells, intestinal cells, etc., were submitted to the same tests. When very small amounts of the extract from these cells (*i. e.*, amounts proportional to those of œnocyte extract) were used, it was impossible to determine whether a pink color had developed or not.

Conclusion.

I conclude from the preceding experiments that the œnocytes, which have been regarded by previous investigators as glands, secrete oxidizing enzymes. I do not know whether this is their only function, but it is certainly one of them. At any rate they do not secrete a fat-splitting enzyme. Since these cells, hanging loosely to the tracheæ, lie free in the blood, the enzymes which they secrete may activate the oxygen of the body towards combustion. That the cells actually secrete is indicated by the fact that numerous observers, myself included, have detected microscopical exudations around the periphery of the cytoplasm, especially at times when the nucleus is greatly ramified, and therefore manifesting its great activity.

Exactly what relation the œnocytes bear to the tracheæ, I am unable to say. I saw no definite attachment, but am inclined to believe that a relation exists and that through this the oxydases, one branch of the group of oxidizing enzymes, are able to get their molecular oxygen with the formation of peroxides. It must, moreover, be remembered that Wheeler, in 1892, found the œnocytes of phryganeid larvæ to be provided with delicate processes which are attached to the tracheal hypodermis. That may, of course, be simply a means for attachment and have nothing to do with the passage of oxygen from one to the other. The location of the œnocytes may be purely due to the absence of certain mechanical forces. Wheeler found that "the œnocytes originate by delamination or immigration from the ectoderm, just caudad to the tracheal involutions and after their differentiation from the primitive ectoderm never divide, but gradually increase in size." Since they never divide after differentiation, the me-

chanical forces of cell division being absent, they are not able to be carried far from their starting point. Moreover, since there are no other growing tissues between them and the ectoderm, except a few small muscles and connective tissue, they are not pressed or shoved out of the way very much, and are so able to retain their original position. I am inclined, however, to the former view, that there is a definite functional relation between these glandular cells and the tracheæ.

That the œnocytes in *Zeuzera pyrina* are so large is probably due to the prolonged larval stage of this insect. As previously stated, it remains a larva three years, during which time it eats ravenously, and grows very heavy, acquiring an enormous amount of fat. Naturally, since it eats so much, and stores up so much reserve food, it has a great deal of material to oxidize, and consequently needs a large supply of oxidizing enzymes. A comparison with other insects would lead me to this view, for those having a short larval stage like the Dipteran larvæ, have much smaller œnocytes in comparison with the remainder of the body than forms with a prolonged larval life, like the phryganeid larvæ, for example.

Before closing, I wish to express my thanks to Professor William M. Wheeler for the kindly advice and encouragement which he has given me at all times. I also wish to thank my father, Dr. Charles Glaser, of Baltimore, for many valuable technical points.

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